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Study of insulin resistance in cybrid cells harboring diabetes-susceptible and diabetes-protective mitochondrial haplogroups

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ABSTRACT

Aim: This study aims to elucidate the independent role of mitochondria in the pathogenesis of insulin resistance (IR).
Methods: Cybrids derived from 143B osteosarcoma cell line and harboring the same nuclear DNA but different mitochondrial haplogroups were studied. Cybrid B4 (the major diabetes-susceptible haplogroup in Chinese population), cybrid D4 (the major diabetes-resistant haplogroup in Chinese population) and cybrid N9 (the diabetes-resistant haplogroup in Japanese population) were cultured in a medium containing 25 mM glucose and stimulated with 0 μM, 0.1 μM, and 1.0 μM insulin. We compared the insulin activation of PI3K–Akt (glucose uptake) and ERK–MAPK (pro-inflammation) signaling pathways, intracellular and mitochondrial oxidative stress (DCF and MitoSOX Red), and their responses to the antioxidant *N*-acetylcysteine (NAC).
Results: Upon insulin treatment, the translocation of cytoplasmic GLUT1/GLUT4 to the cell membrane in cybrid D4 and N9 cells increased significantly, whereas the changes in B4 cells were not or less significant. On the contrary, the ratio of insulin-induced JNK and P38 to Akt phosphorylation was significantly greater in cybrid B4 cells than in cybrid D4 and N9 cells. The levels of DCF and MitoSOX Red, which are indicative of the oxidative stress, were significantly higher in the B4 cells in basal conditions and after insulin treatment. Following treatment with the antioxidant NAC, cybrid B4 cells showed significantly reduced insulin-induced phosphorylation of P38 and increased GLUT1/GLUT4 translocation to the cell membrane, suggesting that NAC may divert insulin signaling from pro-inflammation to glucose uptake.
Conclusions: Mitochondria play an independent role in the pathogenesis of IR, possibly through altered production of intracellular ROS.

1. Introduction

The link between the occurrence of diabetes and mitochondrial genetic variation such as point mutation, deletion and duplication is well defined. However, even the commonest A3243G mutation has been found in only 1.5% among the idiopathic diabetes forms (Gerbitz et al., 1995). This is too rare to account for a major cause of diabetes.

The role of mitochondria in the common type 2 diabetes (T2DM), which has become a world-wide disease, has not been elucidated yet. Insulin resistance (IR) plays a central role in the pathogenesis of T2DM. The association between mitochondrial dysfunction and IR has been recognized; in T2DM patients, mitochondrial dysfunction is related to lower mitochondrial content, reduced intrinsic mitochondrial respiration in skeletal muscles, and reduced muscle ATP production (Heilbronn et al., 2007; Petersen et al., 2004; Phielix et al., 2008; Schrauwen-Hinderling et al., 2007).

Since 1998, a common variant at bp 16189 (T → C transition) in the control region of mtDNA has been found to be associated with raised fasting insulin levels in men, in whom the link between small birth size and impaired glucose tolerance at age 64 was confirmed (Casteels et al., 1999; Poulton et al., 1998). Moreover, a population-based case–control study in Cambridgeshire, UK demonstrated a significant association between the 16189 variant and T2DM (Poulton et al., 2002). Our group has found that in the Chinese population the common variant at bp 16189 was associated with impaired glucose tolerance and T2DM (Liou et al., 2007). It is hypothesized that a T-to-C transition at np 16189, which causes a homopolymeric tract of cytosines at np 16184–16193,

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may cause a defect in the replication process of mtDNA (Liou et al., 2010; Yasukawa et al., 2005). We later found the combined diabetogenic effect of variants in the control region (bp 16189 T → C transition, poly C at nucleotides 16184 to 16193) and a single nucleotide polymorphism (SNP) in the coding region (10398A allele) of mtDNA. These mtDNA haplogroups have a better predictive value of diabetes than a single 16189 variant.

mtDNA haplogroups have been defined on the basis of specific SNPs that are scattered throughout the mitochondrial genome. Phylogenetically related haplotypes have been grouped together to form haplogroups that display a region-specific distribution (Santoro et al., 2006). Recent evidence suggests that mtDNA haplogroups have functional consequences; they are linked to longevity (Rose et al., 2001), sperm motility (Darvishi et al., 2007; Ruiz-Pesini et al., 2000), certain types of cancer (Darvishi et al., 2007), and the risk of individuals developing specific late-onset neurodegenerative diseases (Pyle et al., 2005), stroke (Chinnery et al., 2010), coronary artery disease, and DM complications (Feder et al., 2008). In the Chinese and Japanese populations (Liou et al., 2012), the diabetes-susceptible haplogroup (B4) harbors 10398A and the poly C tract, while the diabetes-resistant haplogroups (D4, N9) harbor 10398G and without the poly C tract. Mitochondrial cybrid cells harboring different mtDNA haplogroups with the same nuclear genetic background are a unique model to survey the possible molecular pathways leading to IR. The diabetes-susceptible haplogroup (B4) and diabetes-protective haplogroups (D4, N9) have been proved to exhibit discriminating tolerance against oxidative stress (Lin et al., 2012).

Insulin functions by binding to its cognate cell membrane receptor which on activation by autophosphorylation recruits and further activates several signaling intermediates, leading downstream to the activation of the PI3-kinase–Akt pathway and ERK–MAPK pathway (Samani et al., 2007). Several independent reports indicate that the activation of the PI3-kinase–Akt pathway most often promotes the effects of insulin on acute cellular metabolism as well as cell growth and protein synthesis, whereas activation of the ERK–MAPK pathway promotes cell proliferation and chronic inflammation (Avruch, 1998). Therefore, this study aims to compare the insulin signaling pathways among the three cybrids, their mtDNA derived from individuals showing different phenotypes of glucose metabolism.

2. Methods and materials

2.1. Cell culture and reagents

Cybrids were derived from the 143B osteosarcoma cell line harboring the same nuclear DNA, but containing different mitochondrial haplogroups. Cybrid B4 (the major diabetes-susceptible haplogroup in Chinese population), cybrid D4 (the major diabetes-resistant haplogroup in Chinese population), and cybrid N9 (the diabetes-resistant haplogroup in Japanese population) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco BRL, USA), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, USA) in 5% CO_2 at 37 °C. The cells were sub-cultured by treating with trypsin (0.05%)–EDTA (1:5000; Gibco BRL, USA). The attached cells displayed cobblestone morphology on reaching confluence. Insulin and NAC (*N*-acetyl-L-cysteine) were obtained from Sigma-Aldrich Co. LLC.

2.2. Isolation of nuclear and membrane extracts

The nuclear and membranes were isolated as previously described (Shi et al., 2007). Briefly, B4, D4, and N9 cells were plated in 15-cm dish plates (Nunc, Denmark) and seeded at a density of 1×10^7 cells per dish. After overnight incubation, the cells were serum starved for 16 h and then treated with a medium containing 25 mM glucose and stimulated with 0, 0.1, and 1.0 μM insulin for 30 min. The harvested cells were resuspended in 500 μl ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 tablet of the protease inhibitor mixture [Roche, Milan, Italy]). The cells were disrupted with 50 strokes of a tight-fitting Dounce homogenizer (Sango, Beijing, China). The homogenate was checked under an Olympus phase contrast microscope (Tokyo, Japan) to confirm complete disruption after which it was centrifuged at 8000 *g* for 10 min to remove the nuclei and mitochondria. The supernatant was re-centrifuged at 100,000 *g* for 30 min. The membrane fraction, obtained as a pellet, was dissolved in 200 μl hypotonic buffer. The membrane proteins were released by treating it with 1% Triton X-100 for 1 h.

2.3. Immunofluorescence analysis

B4, D4, and N9 cells were plated at a density of 8×10^4 cells per well in 12-well plates (Nunc, Denmark). After overnight incubation, the cells were serum starved for 16 h followed by treatment with a nutrient medium containing 25 mM glucose and stimulation with 0, 0.1 and 1.0 μM insulin for 30 min. The cells were then fixed in 4% paraformaldehyde and permeabilized using a buffer containing 1% bovine serum albumin and 0.1% Triton X-100 in PBS and further incubated with anti-GLUT1 (1:100 dilution from Santa Cruz Biotechnology) and anti-GLUT4 (1:100 dilution from Santa Cruz Biotechnology) for 2 h, followed by incubation with Alexa 488- or 546-conjugated secondary antibody for 1 h at room temperature and nuclear staining with DAPI. The slides were visualized under a fluorescence microscope after mounting the cells in Fluoromount media (Sigma-Aldrich Co. LLC).

2.4. Western blot analysis

The cells were plated at a density of 2×10^6 cells per well in 6-well plates (Nunc, Denmark). Following an overnight incubation, the cells were serum starved for 16 h after which they were treated with a medium containing 25 mM glucose and stimulated with 0, 0.1 and 1.0 μM insulin for 30 min. The cells were harvested after which their protein extract was isolated using a buffer containing 150 mM NaCl, 50 mM HEPES pH 7, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, and a protease inhibitor. The proteins were separated via SDS-PAGE by using an 8–10% polyacrylamide gel, and then they were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore) by using a blotting apparatus. The membrane was blocked using 5% milk in TBS-T for 1 h at room temperature, and then incubated overnight at 4 °C with antibodies against anti-GLUT1 (1:1000 dilution from Santa Cruz Biotechnology), anti-GLUT4 (1:1000 dilution from Santa Cruz Biotechnology), anti-pIRS1 (Y896) (1:1000 dilution from Epitomics, Inc.), anti-IRS1 (1:2000 dilution from Merck Millipore), p-Akt (S473) (1:1000 dilution from Santa Cruz Biotechnology), anti-Akt (1:1000 dilution from Santa Cruz Biotechnology), anti-p-P38 (T180/Y182) (1:1000 dilution from Cell Signaling Technology), anti-P38 (1:1000 dilution from Cell Signaling Technology), anti-p-JNK (T183/Y185) (1:1000 dilution from Cell Signaling Technology), anti-JNK (1:1000 dilution from Cell Signaling Technology) and anti-β-actin (1:50 000 dilution from Merck Millipore). Further, following conjugation of the secondary antibody with HRP for 60 min, the signals on the membrane were detected using ECL-plus luminal solution (Advansta, USA) and exposed to an X-ray film for autoradiogram.

2.5. Detection of intracellular ROS by flow cytometry and fluorescence analysis

The oxidation products in the cells were evaluated by measuring the levels of hydrogen peroxide (H_2O_2) using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma). This probe is accumulated by cells and hydrolyzed by the cytoplasmic esterases to 2′,7′-dichlorofluorescin